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NEW TECHNOLOGY DISCLOSURE

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1. CAMPUS SUBMITTING THIS DISCLOSURE
   SUNY - HEALTH SCIENCE CENTER AT BROOKLYN

2. TITLE
   "Mutant T7 RNA Polymerases with Improved Processivity and Fidelity"

3. KEY WORDS
   RNA Polymerase/T7/T3/SP6/Bacteriophage/RNA Probes/Hybridization/Expression Vectors

4. TYPE
   ☒ Invention ☐ Software ☐ Video ☐ Other _____

5. PRIMARY CONTACT *(among developers/inventors)*
   William T. McAllister, Ph.D.

6. DATE OF CONCEPTION / /

7. SPONSORSHIP
   ☐ United States Government ☐ Private Industry ☐ SUNY ☐ Personal
   ☐ Other _____

Name of Sponsor(s)	Research Foundation or Campus Account Number	Sponsored Assigned Identification Number
National Institutes of Health	412-2593 D	GM38147

[redacted]

8. PUBLIC DISCLOSURE

Previous reports in the literature

Mutant polymerases that fail to terminate: We have previously reported that mutations in the region of 178-179 fail to recognize one type of terminator (the PTH terminator) [Macdonald, L.E., et al. (1993) *J. Mol. Biol.* **232**, 1030-1047, Macdonald, L.E., et al. (1994) *J. Mol. Biol.* **238**, 145-158]. However, it was unknown whether the mutant RNAP would fail to recognize other termination signals, nor was it known whether other mutations in nearby or distal residues of the RNAP might give rise to the same phenotype.

Mutant polymerases that don't initiate at 3' ends: Lyakhov, et al. (1992; *Molekuliarnaia Biologiia* **26**, 679-687) had previously disclosed that del 72-173 exhibited this desirable property; however, it was not known whether other residues in the same region or distal regions might give rise to the same phenotype. Furthermore, it was not known (and unexpected) that this phenotype would be related to the one described above, and vice versa.

Public Presentations

Some properties of the first type of mutant (del 172-173) were reported in a poster at a FASEB meeting on "Transcription Inhibition" in Saxton River, Vermont, July 15-20, 1995. Some were published in an article by Macdonald et al. (1995) and some are described in the attached manuscript, which has been submitted for publication [Lyakhov, D.L., et al. (1996)].

9. BRIEF TECHNICAL CONFIDENTIAL DESCRIPTION *(including its unique features) ATTACH ANY MANUSCRIPTS, REVIEWS, PAPERS, DIAGRAMS, CHARTS, ETC.*

We have identified a region of T7 RNAP (amino acid residues 163 to 179) in which substitution or deletion of certain residues eliminates properties 1 and 2 above (that is, premature termination at a variety of signals, and production of anomalous products from templatres having protruding 3' ends). Among the mutations that we have characterized that give rise to this property are: del178-179 (deletion of amino acid residues 178 and 179), del172-173; R173C (substitution of cysteine for the arginine residue usually found a position 173); and del163-164. Other substitutions in this region that do not give rise to the desired phenotyupe include ins173G and ins178G (insertion of glycine residues at positions 173 and 178), suggesting that not all changes in this region of the RNAP give rise to the desired phenotype. We believe that this region of the RNAP is a surface exposed basic loop which may play a role in binding to the nascent RNA, facilitating the isomerization of the RNAP from an unstable initiation complex to a stable elongation complex and its reversal (hence its role in termination).

Although we have characterized these specific mutations, it is possible (and is likely) that other mutations elsewhere in the polymerase or in the same regions that we have identified may also give rise to enzymes with similar properties.

10. PROTOTYPES AND/OR SAMPLES

a. Is a working prototype available for demonstration? ☐ Yes ☐ No ☐ N/A

b. Are samples (e.g. compounds) available for testing? ☐ Yes ☐ No ☐ N/A

11. ADVANTAGES OF THE TECHNOLOGY *(relative to existing technology)*

The improved enzymes that we have constructed do not carry out two side reactions that are problematic with currently available enzymes. We expect that the improved enzymes will rapidly supplant the existing enzymes in the marketplace, for nearly all applications.

12. POSSIBLE DISADVANTAGE(S) OF THE TECHNOLOGY *(relative to existing technology)*

None that we are aware of.

13. NON-CONFIDENTIAL DESCRIPTION OF THE TECHNOLOGY
(Indicate applications and advantages — for marketing purposes)

T7 RNA polymerase (RNAP), and the RNAPs encoded by related phages (e.g., T3, SP6) are widely utilized in vitro for the synthesis of RNA for probes, physical studies, and for other purposes, and in vivo as the basis for expression vectors in both procaryotic and eukaryotic cells.

Although the phage RNAPs are highly efficient, and exhibit a high degree of specificity, there are a number of side reactions that hinder the use of these enzymes under certain circumstances. These undesirable side reactions include:

1) Premature termination of the RNAP within the transcription unit, preventing the synthesis of a full-length product [Mead, D.A. et al. (1986) Prot. Eng. 1,67-74; Macdonald, L.E., et al. (1993) J. Mol. Biol 232,1030-1047; (Macdonald, L.E., et al.(1994) J. Mol. Biol. 238,145-158];

2) Production of transcription products which do not arise from initiation at the phage promoter sequence; these products are seen particularly on templates with protruding 3' ends[Schenborn, E.T. and Mierendorf, R.C., Jr. (1985) Nuc. Acids Res. 13,6223-6236; Lyakhov, D.L. et al. (1992), Molekuliarnaia Biologiia 26,679-697].

Here, we describe the construction of mutant T7 RNAPs which do not carry out these side reactions, but continue to exhnibit normal activity, specificity, and processivity. Although best characterized in the case of T7 RNAP, we have created similar mutations in T7 RNAP. To facilitate purification of the RNAPs, the mutant enzymes have been cloned into a histidine-tagged expression vector.

14. LIST COMPANIES THAT YOU BELIEVE WOULD BE INTERESTED IN COMMERCIALIZING THE TECHNOLOGY

Company Name	Contact (if any)	Location/Telephone Number
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	
[redacted]	[redacted]	

Any company that markets phage RNA polymerases (e.g. T3, T7, SP6) or expression vectors based upon these RNA polymerases. Copies of relevant pages from the Cold Spring Harbor Source Book which lists such companies are attached.

15. SIGNED BY DEVELOPER(S) WITNESS(ES)

a. Name: Dr. Mr. Ms. William T. McAllister
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(Attach Additional Sheets If There Are More Developers) (See attached Page 4a)

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☐ Date of Complete Disclosure: _____

* Drs. Macdonald and Durbin have moved since performance of research. Their home addresses listed above are current.

15. SIGNED BY DEVELOPER(S) AND WITNESS(ES)

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(Attach Additional Sheets If There Are More Developers)

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☐ Date of Complete Disclosure:

*Dr. He has moved since performance of research. Home addressed listed above is correct.

R1215

DEPARTMENT OF MICROBIOLOGY & IMMUNOLOGY
MORSE INSTITUTE FOR MOLECULAR GENETICS

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February 5, 1997

Dr. Albert E. Muir
Licensing Coordinator
Research Foundation Technology Transfer Office
P.O. Box 9
Albany, New York 12201-0009

TECHNOLOGY TRANSFER OFFICE
APR 21 1997
The Research Foundation of State University of New York

Dear Dr. Muir:

Thank you for taking the time to talk to me yesterday concerning licensing and patenting of phage RNA polymerase mutants with altered termination properties. A copy of a patent disclosure which describes this invention is attached. We are still awaiting the signature from one of the inventors, and thus the copy that I am enclosing is not the originial, which will follow shortly.

As I indicated to you, we have received a number of inquiries from companies that wish to market these enzymes. The improved enzymes will rapidly replace use of unmodified forms of the phage RNA polymerases which are covered by patents held by UMDNJ (T3 RNA polymerase) and Brookhaven Laboratories (T7 RNA polymerase). It is urgent this matter be addressed as soon as possible, as we have submitted a publication which describes the properties of these mutants, and this has been accepted for publication (see attached manuscript). Note that on page 16 of this manuscript, the practical application and utility of these enzymes are specifically addressed.

[redacted]

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[redacted]

I greatly appreciate your assistance in this matter.

Sincerely,

W. T. McAllister

William T. McAllister, Ph.D.
Professor and Chairman

WTM:js
Encls.

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Mutant T7 RNA Polymerases with Altered Termination Properties

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Keywords: RNA binding/T7 lysozyme/concatamer junction/packaging/phage RNA polymerase/promoter binding

Running Title: Mutant T7 RNA Polymerases

ABSTRACT

We have identified mutants of T7 RNA polymerase (RNAP) that are altered in their ability to pause or terminate at a variety of signals. These signals include a terminator found fortuitously in the human preproparathyroid hormone (PTH) gene, a pause site found in the concatamer junction (CJ) of replicating T7 DNA, and termination signals that are also utilized by *E. coli* RNAP (*e.g.*, *rrn*B T1 and T2). Whereas the mutant enzymes terminate normally at the late terminator in T7 DNA (TΦ) and *rrn*B T2, they fail to terminate at *one* of the termination sites of *rrn*B T1, and also fail to recognize the PTH and CJ signals. The mutant enzymes exhibit normal processivity on linear templates, but show a slightly reduced processivity on supercoiled templates and terminate more efficiently when synthesizing poly(U) tracts. The mutant enzymes also show a decreased tendency to produce aberrant transcription products from DNA templates having protruding 3'-ends.

T7 lysozyme (an inhibitor of T7 RNAP) has been shown to exert its action by preventing the transition of the RNAP from an unstable initiation complex (IC) to a stable elongation complex (EC) (Zhang & Studier, 1996). We have found that T7 lysozyme enhances recognition of CJ by WT T7 RNAP, and that mutant T7 RNAPs that show increased sensitivity to lysozyme show enhanced recognition of this signal, even in the absence of lysozyme. These results, together with the observation that the mutations that result in the termination-deficient phenotype affect a region of the RNAP that has been implicated in RNA binding and upstream promoter contacts, support the hypothesis that, in some cases, termination represents a reversal of the events that occur during initiation.

INTRODUCTION

T7 RNA polymerase (RNAP) recognizes at least two types of termination signal. Class I terminators are typified by the signal that occurs within the late region of the bacteriophage T7 genome (TΦ), which encodes an RNA that has the potential to form a stem-loop structure followed by a run of U-residues (see Fig. 1) (Dunn & Studier, 1983). These structural features are reminiscent of termination signals utilized by *E. coli* RNA polymerase, and a number of bacterial termination signals have been shown to terminate T7 RNAP (Macdonald *et al.*, 1993; Christiansen, 1988; Jeng *et al.*, 1990; this work), suggesting that there may be a common mechanism of termination for these two enzymes. A second type of termination signal (class II) was first identified in the cloned human preproparathyroid hormone (PTH) gene (Mead *et al.*, 1986). This signal does not encode an RNA with an equivalent stem-loop structure (Fig. 1) yet leads to efficient termination by the phage RNAP (Mead *et al.*, 1986; Macdonald *et al.*, 1993).

In earlier work, we noted that a modified form of T7 RNAP that is proteolytically cleaved between residues 178 and 179 (20K/80K enzyme) exhibits altered termination properties (Macdonald *et al.*, 1993). Although the 20K/80K enzyme is less processive than the intact RNAP (giving rise to products with a shorter average chain length; Ikeda & Richardson, 1987) it shows a slightly reduced efficiency of termination at TΦ and fails to recognize the PTH terminator entirely (Macdonald *et al.*, 1993). These results indicate that termination is not due to a generalized decrease in processivity (otherwise, an enzyme with reduced processivity would exhibit an increase in termination efficiency), and suggest that the PTH and TΦ signals lead to termination by nonequivalent mechanisms.

It has been proposed that termination by T7 RNAP involves a reversal of the process that leads from an unstable initiation complex (IC) to a stable elongation complex (EC) (Sousa *et al.*, 1992; Macdonald *et al.*, 1994; McAllister, 1996). In this model, the initial association of the nascent RNA with the product binding site triggers an isomerization event that stabilizes the ternary complex, results in loss of upstream promoter contacts, and allows the RNAP to move away from the promoter (ibid). During elongation, changes in the nature of the association of the nascent RNA with the product binding site are thought to trigger the reversal of this isomerization, resulting in dissociation of the EC. A number of

observations support this proposal. It had previously been observed that the 20K/80K enzyme is less able to bind single-stranded RNA than the intact RNAP, and that further degradation of the 20kDa N-terminal moiety of the RNAP with trypsin results in a form of the enzyme (80K) that fails to bind single-stranded RNA entirely and is blocked in the formation of a stable elongation complex (Muller *et al.*, 1988). These findings led to the proposal that an RNA-binding site is present in the N-terminal portion of the RNAP, and that association of the nascent RNA with this site is required to establish and maintain the EC (ibid). As noted above, in addition to its altered properties with regard to RNA binding and initiation, the proteolytically cleaved enzyme also exhibits altered termination properties.

The positions of the proteolytic cleavage sites in T7 RNAP have been reported to be between adjacent basic residues K172 and R173 (Ikeda & Richardson, 1987) or K178 and K179 (Grodberg & Dunn, 1988; Muller *et al.*, 1988). In earlier work, we characterized mutant T7 RNAPs that had been specifically altered in the region around residues 178 and 179, and showed that these enzymes fail to terminate at the PTH signal (Macdonald *et al.*, 1994). We have now extended these observations to show that mutant RNAPs that are affected at other basic residues in the same region fail to recognize the PTH signal, as well as a number of other pause/termination signals.

It has been reported that other mutant RNAPs that are altered in this region (i.e., R173C) are transcriptionally active, but are unable to complement the growth of T7 phage that do not encode a functional RNA polymerase (Zhang & Studier, 1995; Zhang, 1995). The block in phage replication in such infected cells is at the level of processing of the concatamer junction (CJ) and packaging of mature phage DNA into phage capsids (ibid). As it is possible that pausing or termination of the RNAP in the CJ might be important for these processes, we asked whether the wild type and the termination-defective RNAPs exhibit different properties when transcribing through the CJ. We found that a signal in the CJ functions as a pause site for T7 RNAP, and that the termination-defective RNAPs fail to utilize this signal.

T7 lysozyme (an inhibitor of T7 RNAP; Moffatt & Studier, 1987; Ikeda & Bailey, 1992) exerts its action by preventing the transition of the enzyme to a productive elongation complex (Zhang & Studier, 1996; Zhang, 1995). As it has been proposed that termination represents a reversal of the process that

leads from an IC to an EC, we were curious to determine whether lysozyme might affect termination by the wild-type enzyme, and whether RNAP mutants that showed increased sensitivity to lysozyme might exhibit altered termination properties. We have found that lysozyme stimulates pausing or termination by wild type (WT) T7 RNAP at CJ, and that mutants of T7 RNAP that show increased sensitivity to lysozyme show enhanced recognition of this signal, even in the absence of lysozyme.

The phage RNAPs are known to synthesize aberrant products from templates that have protruding 3' ends (Schenborn & Mierendorf, Jr. 1985; Lyakhov *et al.*, 1992). It had been observed that a mutant T7 RNAP in which residues 172-173 are deleted gives rise to lower amounts of these aberrant products (Lyakhov *et al.*, 1992). A comparison with other mutant enzymes that are affected in this region shows that this is a common property among the termination-deficient RNA polymerases. This may be of practical significance to investigators who wish to synthesize more homogeneous RNA products.

MATERIALS AND METHODS

Enzyme purification. Some of the mutant RNAPs characterized in this work have been described earlier (see Table I and Zhang & Studier, 1995; Zhang, 1995). Others were created in the course of this work, as described in He (1996) and He *et al.* (1997b) using the following DNA primers for mutagenesis:

pDL60: 5'-GGAACAGCTTAACCATGGGCAAGTCTAC-3'

pBH173: 5'-GACCTTGAAGCTAAGCACTTTAAAGCAAACGTTGAGGAACAACTC-3'

pBH187: 5'-CTTGAAGCTAAGCACTTCGCGAAAAACGTTGAGGAACAA-3'

pBH205: 5'-GACCTTGAAGCTAAGCACTTCAATGTTGAGGAACAACTCAACAAG-3'

pBH180: 5'-AAGAAAAACGTTGAGGAGGCCTTGAACAAGCGCGTAGGGCAC-3"

pRC10 was constructed by subcloning the T7 RNAP gene from X92 (Zhang & Studier, 1995) into pBH161 (He *et al.*, 1997b). pBH200 was constructed by subcloning a 500 bp XhoI-NruI partial digestion fragment from pBH187 into the XhoI-StuI sites of pBH180. T7 and T3 RNAPs were purified from an overexpressing strain of *E. coli* as described by He *et al.* (1997b). SP6 RNAP was purchased from New England Biolabs. T7 lysozyme was overexpressed and purified as described by Cheng *et al.* (1994).

Plasmid construction and template preparation. Plasmid templates were constructed by standard cloning procedures (Sambrook *et al.*,1989); complete sequences are available upon request (see Fig. 2). pDL44 and pDL42, which contain the T7-TΦ and PTH terminators downstream from a consensus T7 promoter, were constructed in three steps. First, the polylinker of pSL1180 was cloned into the XmaI-HindIII sites of pKK232-8 (both plasmids are from Pharmacia Biotech) to generate pDL38. A 258 bp EcoRI-NheI fragment containing the T7 promoter was excised from pGD13 (Diaz *et al.*, 1996) and cloned into the MunI-NheI sites of pDL38 to generate pDL39. To generate pDL44, a 222 bp EagI-Acc65I fragment from pLM24 (Macdonald *et al.*, 1994) that contains T7-TΦ was cloned into the EagI and BsrGI sites of pDL39. To generate pDL42, a 203 bp Acc65I-XbaI fragment from pLM44 (Macdonald *et*

al., 1994) that contains the PTH signal was cloned into the Acc65I and BlnI sites of pDL39. After digestion with AflII, pDL44 serves as a transcription template which gives rise to terminated and run-off products of 416 nt and 561 nt, respectively, while pDL42 gives rise to terminated and run-off products of 349 nt and 541 nt. Plasmid pT7-SC, which contains the *rrn*B T1 and T2 terminators under control of T7 Φ10, was obtained from United States Biochemicals; when digested with DraI this template gives rise to run-off and terminated products as indicated in Fig. 2. Plasmid pAR2813 (Zhang & Studier, 1996) contains a 1.3 kb fragment of phage T7 DNA that includes the T7 promoter ΦOR and sequences surrounding the concatamer junction; when digested with HindIII this template gives rise to a 1178 nt run-off product. Plasmid pLM22 contains a $(dA)_{40}$ tract downstream from a T7 promoter (Macdonald *et al.*, 1993; see Fig 6A). All plasmids were purified by isopycnic banding in CsCl gradients (Sambrook *et al.*, 1989).

Transcription reactions. Unless otherwise noted, transcription reactions were carried out in a volume of 20 µl containing 30 mM HEPES, pH 7.8; 100 mM potassium glutamate; 5 mM $Mg(OAc)_2$; 0.25 mM EDTA; 1 mM DTT; 0.05% Tween-20 (Maslak & Martin, 1994); 0.5 mM ATP, CTP, GTP and UTP (Pharmacia, Ultrapure); 1 µCi [alpha -^{32}P]-ATP (specific activity of 800 Ci/mmol; New England Nuclear); 50 ng T7 RNAP; and 1 µg linearized plasmid DNA template. Reactions were preincubated at 37°C for 2 min and RNA synthesis was initiated by addition of template or RNAP, as indicated. After 30 min, 2 µl aliquots were mixed with 5 µl stop buffer (98% deionized formamide; 10 mM EDTA, pH 8.0; 0.025% xylene cyanol FF; 0.025% bromophenol blue) in a MicroSample Plate (Pharmacia Biotech) and heated for 5 min in a 90°-95°C water bath. The products were resolved by electrophoresis in 5-15% (depending on the size of the products) polyacrylamide gels in the presence of 7M urea (Sambrook *et al.*, 1989). The products were visualized by exposure of the gel to a PhosphorImager™ screen (Molecular Dynamics) for 30-60 min. All data were within the linear range of screen response and were analyzed using ImageQuant™ (Molecular Dynamics).

Determination of RNAP processivity. pBluescript II SK+ (Stratagene) was transcribed *in vitro* with T7 RNAP either in linear form (digested with NgoMI to give run-off products greater than 2,600 nt) or in supercoiled form. The reactions (40 µl) contained 0.25 mM GTP, ATP, CTP and UTP,

20 μCi [gamma-^{32} P]-GTP (specific activity 6,000 Ci/mmol; New England Nuclear), and 1 μCi [8,5'-^{3}H] GTP (specific activity 28.4 Ci/mmol; New England Nuclear). After 10 min at 37°C, the incorporation of radioactive substrate into acid-precipitable material was determined as described in He *et al.* (1997b). An estimate of the relative average chain length was determined from the molar ratio of internal label (^{3}H GTP) to 5'-end label (gamma-^{32}P-GTP) (Ikeda & Richardson, 1987).

Lysozyme inhibition assays. Reactions were performed under standard conditions. T7 RNAP was premixed in transcription buffer either with T7 lysozyme (molar ratio T7 RNAP:T7 lysozyme = 1:6) that had been freshly diluted in storage buffer (20 mM $KHPO_4$; pH 7.7; 1 mM EDTA, pH 7.5; 100 mM NaCl; 1 mM DTT; 50% glycerol) or with the same volume of storage buffer. The reactions were preincubated at 37°C for 2 min and RNA synthesis was intitiated by addition of prewarmed DNA template.

RESULTS

Mutant RNA polymerases.

The sites of proteolytic cleavage in T7 RNAP have been reported to be between residues 172-173 or 178-179 (Ikeda & Richardson, 1987; Muller *et al.*, 1988; Grodberg & Dunn, 1988). These residues are predicted to lie within α-helix I (169-183) and adjacent to a solvent-exposed loop (aa 151-168) that links α-helices H and I (Sousa *et al.*, 1993; see Fig. 3). A number of mutant enzymes that are affected in this region have been constructed (Table I). The properties of these enzymes with regard to termination were determined by transcription *in vitro*, as shown in Figure 4 and Table II. All of the enzymes were catalytically active and terminated at TΦ nearly as efficiently as the wild type (WT) RNAP. However some of the mutants showed altered termination properties at other signals, as noted below.

Termination at the PTH terminator.

Many (but not all) RNAP mutants that are affected in the region from residue 163 to 193 fail to recognize the PTH terminator (or show a major reduction in this ability; e.g., *del*163-4). In general, insertions and/or deletion mutations that are expected to alter the structure of this region in a substantial manner resulted in this phenotype, whereas less substantial alterations such as single amino acid substitutions did not. Thus, for example, deletion of residues 163-164 or 172-173 caused reduced termination, but replacement of K172 with G or L, or replacement of K163 or K164 with A, did not. On the other hand, replacement of R173 with C did result in the termination-defective phenotype.

The amino acid sequence from residue 160 to 195 in T7 RNAP is well conserved in the RNAPs from the related bacteriophages T3 and K11, but diverges significantly in SP6 RNAP (Fig. 5). Nonetheless, certain residues such as K164 and R173 are identical in all four RNAPs, or represent conserved substitutions of basic residues (e.g., K179->R in SP6 RNAP). It is therefore noteworthy that the T3 and SP6 RNAPs recognize the PTH terminator with approximately the same efficiency as T7 RNAP (Table II). Moreover, deletion of residues 173-174 in T3 RNAP (which correspond to residues 172-173 in T7 RNAP) results in a mutant T3 RNAP (T3 *del*173-4) that also fails to recognize the PTH terminator (Table II). These results suggest that these conserved residues are important for recognition of this class of terminator, and that a common structural element may be involved. The observation that

individual amino acid substitutions at these key positions did not always result in a change in the termination phenotype suggests either that there may be some redundancy in the function of these residues, or that significant structural alterations (such as those introduced by a deletion mutation) are required to cause this effect. In the discussion that follows, we have considered the mutant T7 RNAP *del*172-3 as the prototype of this class of enzymes.

Termination at *rrn*B T1 and T2.

T7 RNAP has been shown to terminate at sequences such as *rrn*B T1 and T2 that serve as factor-independent termination signals for *E. coli* RNA polymerase (Macdonald *et al.*, 1993; Macdonald *et al.*, 1994; Jeng *et al.*, 1990; Christiansen, 1988). The sites of termination by WT T7 RNAP at the *rrn*B T1 terminator were mapped by Christiansen (1988) to three positions. The first two (site A: C-6650 and U-6652) are on the descending side of a potential stem-loop structure, just before a poly(U) stretch, and the third position (site B: G-6675) lies 15 nt downstream from the base of the putative stem (Fig. 1). Whereas *del*172-3 terminates normally at site A in *rrnB* T1 and at *rrnB* T2, it fails to terminate at site B in *rrnB* T1 (Fig. 4B, C; Table II). Hartvig and Christiansen (1996) have also recently reported that site B in *rrnB T1* behaves as a class II termination signal.

Pausing by T7 RNAP at a signal in the concatamer junction.

A number of T7 RNAP mutants have been identified that are transcriptionally active yet fail to complement the growth of T7 phage that are deficient in the gene that encodes T7 RNAP (Gross *et al.*, 1992; Zhang & Studier, 1995). One such mutant is R173C, which is blocked at the level of processing of the concatamer junction and packaging of the mature phage DNA into the phage capsid (Zhang & Studier, 1995). As it is possible that pausing or termination of the RNAP at CJ may be important for processing and/or packaging, we asked whether R173C and *del*172-3 exhibit differences *in vitro* when transcribing through CJ as compared to the WT enzyme.

T7 DNA replicates as concatamers (that is, head-to-tail repeats of T7 DNA joined by one copy of the 160 bp repeat found at each end of the mature genome). The region around the concatamer junction contains a T7 promoter near the right end of the genome (ΦOR) followed by a series of short repeated sequences, the terminal repeat (TR), and a promoter near the left end of the genome (ΦOL) (Dunn &

Studier, 1983). A portion of the region that contains ΦOR and CJ has been cloned into the plasmid pAR2813 (Zhang & Studier, 1996; see Fig. 2). Transcription of pAR2813 by T7 RNAP *in vitro* reveals a signal within this region that is recognized by WT T7 RNAP but not by del172-3 or R173C (Fig. 4D). In work to be reported elsewhere, we have determined the precise site of termination in CJ as indicated in Figure 1E, and have found that the CJ signal acts as a pause site that does not lead to as efficient release of RNA as is observed at TΦ or the PTH signal (Lyakhov *et al*, unpublished observations; Zhang & Studier, 1996). This behavior is not related to the promoter from which transcription is intiated, as the CJ signal is observed to act as a pause site even when transcription is initiated at a consensus promoter (ibid).

Effects of T7 lysozyme on termination.

T7 lysozyme (an inhibitor of T7 RNAP; Moffatt & Studier, 1987; Ikeda & Bailey, 1992) exerts its action by preventing the isomerization of the enzyme to a productive elongation complex (Zhang & Studier, 1996). The effect of lysozyme on recognition of various termination signals is presented in Table III. Whereas the inhibitor had only a slight effect on termination by WT RNAP at TΦ, PTH, and the *rrn*B terminators, it significantly increased recognition of the CJ signal (from 0.26 to 0.77). The addition of lysozyme, however, did not restore the ability of *del*172-3 to recognize this signal.

In view of effect of lysozyme on recognition of CJ, we asked whether RNAP mutants that show *i*ncreased *s*ensitivity to *l*ysozyme (ISL mutants) exhibit altered recognition of this signal, or of other termination signals. Two ISL mutants -- X19 (A465T) and BG8 (A702T) (Zhang & Studier, 1995; Zhang, 1995) -- show enhanced recognition of the CJ signal even in the absence of lysozyme (Table III). Significantly, X19, which exhibits one of the greatest sensitivities to lysozyme *in vivo* of all ISL mutants (Zhang & Studier, 1995) shows the highest efficiency of termination *in vitro* at all termination signals that we have examined. We also characterized a number of mutant enzymes that show *d*ecreased *s*ensitivity to *l*ysozyme (DSL mutants; Zhang & Studier, 1995) and have found that their termination properties are indistinguishable from those of the WT RNAP at TΦ and the PTH and CJ terminators (data not shown).

Processivity of the *del*172-3 mutant.

It had been reported that the 20K/80K RNAP is less processive than the intact enzyme and gives rise to RNA products of shorter average chain length when transcribing long transcription units (Ikeda & Richardson, 1987). To compare the processivity of the WT and mutant enzymes, we utilized gamma-^{32}P-GTP to label the initiating nucleotide of the RNA and ^{3}H-GTP as an internal label, and determined the ratio of 5' to internal label as a relative indication of the average chain length. Whereas the processivity of *del*172-3 (and other termination-defective RNAPs) is nearly identical to that of the WT enzyme on linear templates, the processivity of *del*172-3 on supercoiled templates is slightly reduced (*ca* 2-fold; data not shown). The latter observation may be of significance, as it has been reported that the 20K/80K enzyme (which exhibits altered termination properties; Macdonald *et. al,* 1994) may be defective in transcript release and/or displacement of the RNA product (Ikeda & Richardson, 1987). The ability of the RNAP to displace the product, or to maintain the integrity of the transcription bubble, may be important to the termination event, and may be affected by the helical stability of the DNA template. A mutant enzyme with altered termination properties may respond differently than the WT enzyme to changes in the helical stability of the template.

Termination in repeating polynucleotide tracts.

Both class I and class II termination signals are characterized by a run of U residues in the nascent RNA, and the question arises as to whether the synthesis of such a product is inherently destabilizing to an elonagating RNAP. In previous work, we showed that WT T7 RNAP will "slide" when it encounters a poly(dA) tract in the template strand, incorporating additional or fewer UMP residues than are encoded by the DNA (Macdonald *et al.*, 1993). The generation of shorter products presumably results from the low stability of rU:dA base pairs, so that the ternary complex may slide forward along a template without incorporating nucleotides. Generation of a longer-than expected product results from backward slippage of the RNA product and subsequent incorporation of additional nucleotides without translocation of the enzyme (ibid). Whereas both phenomena are observed at normal concentrations of UTP (0.4 mM), at lower concentrations of UTP (where the rate of polymerization is reduced) forward slipping predominates, and some termination by the WT enzyme within the poly(dA) tract is observed. The 20K/80K enzyme shows enhanced termination when it encounters such poly(dA) tracts, especially at

low concentrations of UTP. We have explored this phenomenon with WT and mutant enzymes, and have found that both are equally likely to slip (as evidenced by heterogeneous products and a decrease in chain length at low substrate concentrations). However, the mutant enzyme (like the 20K/80K enzyme) terminates more frequently in the poly(dA) tract at low concentrations of UTP (Fig. 6).

Synthesis of aberrant products on templates with protruding 3' termini.

Although the phage RNAPs synthesize aberrant products when transcribing linear templates that have protruding 3' ends (Schenborn & Mierendorf, Jr. 1985; Lyakhov *et al.*, 1992), Lyakhov *et al.* (1992) had previously observed that *del*172-3 failed to synthesize these products. We have found that this is a common property among the termination-defective RNAPs. All of the mutant enzymes that are termination-deficient show a decrease in the production of these aberrant products, and mutant enzymes that are normal with regard to termination continue to show this property (data not shown). The aberrant transcription from templates with protruding 3' termini occurs in a variety of sequence contexts, and is apparently structure-dependent and not sequence-dependent (see Fig. 7).

DISCUSSION

It has been proposed that termination by T7 RNA polymerase involves a reversal of the steps that lead from an unstable initiation complex (IC) to a stable elongation complex (EC) (Sousa *et al.*, 1992; Macdonald *et al.*, 1994; McAllister, 1996). In this model, the association of the nascent RNA with a product binding site in the amino terminal portion of the RNAP during the early stages of initiation triggers an isomerization event that stabilizes the RNAP and allows it to maintain association with the template during processive elongation. This isomerization (like the isomerization that results in the formation of a stable EC by *E. coli* RNA polymerase) is accompanied by changes in the nature of the contacts of the RNAP with upstream regions of the promoter, and by clearance of the RNAP from the promoter. It is thought that continued association of the nascent RNA with the product site is required to maintain the stability of the EC, and that alterations in the nature of this association result in reversal of the isomerization and dissociation of the EC (ibid). The findings in this work that mutations that prevent recognition of at least one class of termination signal (class II) affect a region of the RNAP that is also involved in promoter binding and binding of single stranded RNA (see below) are consistent with this proposal. Nevertheless, the observation that these mutant enzymes continue to recognize class I termination signals suggests that other pathways to termination may exist.

T7 lysozyme has recently been reported to exert its effect by preventing the transition of T7 RNAP from an IC to an EC (Zhang & Studier, 1996). The observation that lysozyme enhances recognition of certain pause/termination signals (such as CJ), and that RNAP mutants that show an increased sensitivity to lysozyme recognize these signals with greater efficiency, are also consistent with the view that termination and the initial isomerization event are related in an inverse manner.

Structural considerations.

The mutations that give rise to the termination-defective RNAPs described in this paper affect a region of the RNA polymerase that has previously been implicated in promoter binding (Tunitskaya *et al.*, 1990; Gross *et al.*, 1992) and binding of single stranded RNA (Muller *et al.*, 1988; He *et al.*, 1997a). Although the structure of this region of the RNAP is less well defined than that of the C-terminal region, currently available evidence suggests that these mutations affect alpha helix I and the loop that connects

alpha helix I to alpha helix H (Sousa *et al.*, 1993; see Fig. 3). This loop projects into the DNA binding cleft, where it could potentially make contact with the promoter. Indeed, insertion of two amino acid residues at position 159 (*ins*159) results in a polymerase that shows reduced promoter binding (Gross *et al.*, 1992) as do substitutions of residues E158 and K160 with alanine (He, 1996). Specific contacts between the RNA polymerase and the bps from -11 to -8 in the promoter have been shown to involve a specificity loop (residues 742 to 773) that also projects into the DNA binding cleft (Raskin *et al.*, 1992; Raskin *et al.*, 1993). Elsewhere, we have noted that the tip of this specificity loop (residue F755) lies within 5 Å of K179, a key residue in the termination-defective phenotype (Macdonald *et al.*, 1994; see Fig. 3). The mutations that we have identified are therefore located in a region of the polymerase that might communicate directly or indirectly with structures that are involved in promoter contacts, as would be expected if they are involved in the initial isomerization event.

During the early stages of initiation, T7 RNA polymerase continuously synthesizes and releases short abortive transcripts 5-11 nt in length before clearing the promoter and forming a stable EC (Martin *et al.*, 1988; Ling *et al.*, 1989; Diaz *et al.*, 1996). Mutations that affect binding of RNA and the release of these abortive products have been mapped to alpha helix H, which lies at the other end of the loop that projects into the DNA binding cleft (He *et al.*, 1997a; see Fig. 3). Thus, many of the elements needed to form a "trigger" that could recognize the synthesis of a nascent RNA or RNA/DNA hybrid and make or break upstream promoter contacts are located in this region of the RNAP. Alterations in the manner in which these structural elements interact, for example as a consequence of changes in the nature of the association of newly synthesized RNA with the product binding site, could be important for isomerization -- both in the forward direction (IC->EC) and its reversal (termination). Elsewhere we have found that *del*172-3 is defective in RNA binding (He, 1996). However, not all RNA polymerase mutants that are defective in RNA binding exhibit the termination-deficient phenotype (most notably E148A; He *et al.*, 1997a; He, 1996) and thus this phenomenon is likely to be complex.

Nature of the termination signal.

The mutations that we have identified affect recognition of the PTH, *rrnB T1*-site B and CJ signals, but do not greatly affect recognition of TΦ. These two types of signals differ in a number of

ways. First, the PTH and CJ signals do not encode an RNA with an apparent stem-loop structure, as is observed at TΦ. Furthermore, whereas the PTH signal operates in a context-independent manner, the efficiency of termination at TΦ is sensitive to sequences that lie upstream from the signal (Macdonald *et al.*, 1994). The latter observation suggests that alternate configurations of the EC may be formed as the polymerase transcribes through the region that precedes the termination site. For example, the ability of the RNAP to recognize TΦ may depend upon the ability of the nascent RNA to form a particular structure as it emerges from the active site and this, in turn, may reflect the opportunities for alternate folding of the RNA conferred by upstream sequences (in a manner that is analogous to attenuation in certain bacterial operons).

It is possible that the CJ and PTH signals exert their effect by altering the shape of the RNA product in some unknown manner, or by inducing a conformation change in the DNA template and/or RNA:DNA hybrid. Whereas TΦ is recognized by T7 RNAP when transcribing a single strand DNA template, the PTH and rrnB T1-site B signals are not (Macdonald *et al.*, 1994; Hartvig & Christiansen, 1996; He *et al.*, unpublished observations). In work to be reported elsewhere, we have found that the CJ, PTH and rrnB T1-site B signals share a common 7 bp DNA sequence that lies approximately 7 nt upstream from the termination site (see Fig. 1). Further studies concerning the important residues in this class of terminators may shed light on this question. It will also be of interest to determine whether other RNAPs, such as *E. coli* RNAP, respond to this signal.

Practical applications.

The phage RNA polymerases are frequently used to synthesize RNA probes or long transcripts from novel templates, and it is not uncommon to encounter termination signals that prevent or inhibit the synthesis of full length products. Mutant RNAPs that fail to recognize certain termination signals, such as those described in this report, may be useful in circumventing this problem and may be superior to the WT enzyme when transcribing problematic or uncharacterized templates. The finding that these mutant RNAPs also fail to make aberrant products from templates with protruding 3' ends makes them even more attractive when there is a requirement for a chemically homogeneous product (Pestova *et al.*, 1996). We do not know the mechanism by which these aberrant products arise. Perhaps the mutant

enzymes have a decreased affinity for protruding 3' ends or a reduced ability to initiate at such termini. Although it had previously been reported that the production of aberrant products is diminished when the salt concentration is increased (D'Alessio, 1987), we have not been able to confirm this finding (Lyakhov, unpublished results).

Wild type T7 RNAP is sensitive to cleavage by the *ompT* protease and by trypsin, and the primary site(s) of cleavage by these enzymes are in the region that is altered in the mutant RNAPs described in this work. However, 178-193WIHM remains sensitive to the *ompT* protease (Dunn, unpublished observations) and *del*172-3 remains sensitive to trypsin (Kochetkov, pers. comm.), presumably as a result of cleavage of these RNAPs at secondary sites.

ACKNOWLEDGEMENTS

Work at SUNY Health Science Center at Brooklyn was supported by NIH grant GM38147. Work at Brookhaven National Laboratories was supported by the Office of Health and Environmental Research of the US Department of Energy. We are grateful to Mr. Ray Castagna for technical assistance, and to Ms. Rita Gould and Roseann Lingeza for secretarial assistance. We thank Drs. Sergei Borokhov and Rui Sousa for thoughtful comments on the manuscript.

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FIGURE LEGENDS

Figure 1. Termination signals

The RNA sequences encoded by the region around each signal, the potential secondary structures in the RNA, and the stabilities of these structures (in kCal, as calculated by RNASE (R.J. Cedergren, D. Gauthert, F. Major; Universite de Montreal)) are presented. The sites of termination by T7 RNAP (if known) are indicated by shaded boxes and bold letters. There are two regions of termination by T7 RNAP at the *rrn*B T1 terminator: site A (C-6650, U-6652) and site B (G-6675) (Christiansen, 1988). The regions in which termination by *E. coli* RNAP occurs in *rrn*B T1 are indicated by a solid line at the tip of the loop and the descending arm of the stem (Sarmientos *et al.*, 1983). The precise site of termination by T7 RNAP at the *rrn*B T2 terminator is not known; however, results in this study indicate that it is likely to be in the region indicated by the double underline. The RNA products encoded by the PTH and CJ termination signals do not form equivalent stem-loop structures; the minimal sequences that are required for terminator function at each of these signals are indicated by open boxes, and the site(s) of termination are indicated by bold letters and shaded boxes (Lyakhov, D. and He, B., unpublished observations). Note that a common 7 nt sequence (5'-AUCUGUU-3') is found in the PTH, CJ, and *rrnB T1*-site B termination signals (Lyakhov, D. and He, B., unpublished observations; Zhang & Studier, 1996; Hartvig & Christiansen, 1996).

Figure 2. Plasmid Templates

Plasmids to be used as transcription templates were constructed as described in Materials and Methods. pDL44 contains the class I terminator TΦ cloned downstream from a consensus T7 promoter (P_{T7}); digestion of this template with *Afl* II prior to transcription results in the production of terminated and run-off products of 416 and 561 nt, respectively. pDL42 contains the class II PTH terminator downstream from a consensus promoter and gives rise to terminated and run-off products as indicated. pT7-SC (United States Biochemicals) carries the *E. coli* ribosomal terminators *rrnB T1* and *rrnB T2* downstream from a consensus promoter, and gives rise to terminated and run-off products as indicated (see Fig. 1 for further details). pAR2813 contains 1.3 kB of T7 DNA that spans the concatamer junction; this element contains ΦOR (a T7 promoter near the right end of the genome), gene *19.5*, the terminal repetition (TR), and a pause/termination signal (solid box) that gives rise to a product of *ca* 720 nt (see Fig. 4D).

Figure 3. Structure of T7 RNAP

The alpha carbon backbone of T7 RNAP is presented (Sousa *et al.*, 1993). The view is into the DNA binding cleft; the "thumb" domain (Bonner *et al.*, 1994) is indicated to facilitate orientation. The specificity loop (residues 742-773) and the region comprising alpha helices H to I (residues 138-183) are highlighted in black. Key residues indicated by filled spheres include: E148 (involved in RNA binding; He *et al.*, 1997a); A159 (involved in promoter binding; Gross *et al.*, 1992); F755 (at the tip of the specificity loop); and K164, K172, and K179 (involved in termination; this work).

Figure 4. Termination by mutant RNAPs *in vitro*

Plasmids containing the termination signals indicated were cleaved with an appropriate restriction enzyme and transcribed with the RNAP indicated (see Fig. 2 and Table I). The RNA products were resolved by electrophoresis in 5% polyacrylamide gels in the presence of 7M urea and visualized by exposure to a PhosphorImager™ screen. The run-off and terminated products are identified in the margin, along with their expected lengths (in nt). The efficiency of termination at each signal was determined by quantitation of the PhosphorImager™ data and is presented in Table II.

Figure 5. Alignment of phage RNAP amino acid sequences

The amino acid sequences of the T7, T3, K11 and SP6 RNAPs (Dunn & Studier, 1983; McGraw *et al.*, 1985; Dietz *et al.*, 1990; Kotani *et al.*, 1987) are aligned. Shaded boxes indicate residues that are identical with T7 RNAP; basic residues (R,K,H) are in boldface; residue numbers are above the line. A potential alignment of this region of the phage RNAPs with mouse DNA polymerase ß (Matsukage *et al.*, 1987) and the yeast mitochondrial RNAP (Masters *et al.*, 1987) has previously been noted (Lyakhov *et al.*, 1992).

Figure 6. Termination and slippage in poly(dA) tracts

Panel A. Plasmid pLM22 contains a $(dA)_{40}$ tract located 32 base pairs downstream from a T7 promoter, followed by 47 base pairs of DNA before the occurrence of an *Eag* I site (Macdonald *et al.*, 1993). Digestion of this plasmid with *Eag* I and transcription by T7 RNAP *in vitro* is expected to give rise to a transcript of 119 nt. Panel B. This template was transcribed *in vitro* in the presence of varying concentrations of UTP (2.3-500 μM) and fixed amounts of ATP, CTP and GTP (500 μM). The products were resolved by electrophoresis in a 15% polyacrylamide gel in the presence of 7M urea. Backward

slippage of the enzyme in the presence of a high concentration of UTP is indicated by a diffuse RNA product band in the region of 119 nt and greater; forward slippage of the enzyme is revealed by products shorter than the expected run-off transcript (in the range of *ca* 90 nt); termination of the transcript in the poly(dA) tract is indicated by the presence of products 40-60 nt in length (ibid). The positions of RNA size markers are indicated in the margin.

Figure 7. Synthesis of aberrant products from templates with protruding 3' ends

Plasmid pBluescript II KS+ (Stratagene) was digested with *Bsp*120I or *Apa* I (which recognize the sequence GGGCCC and generate 5' or 3' protruding ends, respectively) or with *Acc* 65I or *Kpn* I (which recognize the sequence GGTACC and generate 5' and 3' protruding ends, respectively). The templates were transcribed with WT T7 RNAP or *del*172-3, and the products were resolved by electrophoresis in 6% polyacrylamide gels in the presence of 7M urea

Table I. Mutant RNA Polymerases

ID	Genotype	Plasmid	Amino Acid Sequence[1]	Ref.[2]
T7 RNAP:			165 170 175 180 185 190 195	
WT	*wild type*	pAR1219	FKKNVEEQLNKRVGHVYKKAFMQVVEADMLSKGLL	1
K163A	K163A	pBH187[3]	F**A**KNVEEQLNKRVGHVYKKAFMQVVEADMLSKGLL	2
K164A	K164A	pBH173[3]	FK**A**NVEEQLNKRVGHVYKKAFMQVVEADMLSKGLL	2
del163-4	*del*(K163, K164)	pBH205[3]	F - - NVEEQLNKRVGHVYKKAFMQVVEADMLSKGLL	2
163-9A	*subs*(163-169)->A	pBH200[3]	F **A** LNKRVGHVYKKAFMQVVEADMLSKGLL	2
K172G	K172G	pK172G	FKKNVEEQLN**G**RVGHVYKKAFMQVVEADMLSKGLL	3
K172L	K172L	pK172L	FKKNVEEQLN**L**RVGHVYKKAFMQVVEADMLSKGLL	3
R173C	R173C	pRC10[3]	FKKNVEEQLNK**C**VGHVYKKAFMQVVEADMLSKGLL	4
del172-3	*del*(K172, R173)	p 172-3	FKKNVEEQLN - - VGHVYKKAFMQVVEADMLSKGLL	3
178-193WIHM	*subs*(178-193)->WIHM	pAR3207	FKKNVEEQLNKRVGHVY **W I H M** LL	5
*ins*G181	*ins*G181	pAR3239	FKKNVEEQLNKRVGHVYKK**G**AFMQVVEADMLSKGLL	5
*ins*G180	*ins*G180	pAR3280	FKKNVEEQLNKRVGHVYK**G**KAFMQVVEADMLSKGLL	5
T3 RNAP:			165 170 175 180 185 190 195	
WT	*wild type*	pCM56	FKKHVEEQLNKRHGQVYKKAFMQVVEADMIGRGLL	6
del173-4	*del*(K173, R174)	pDL60[3]	FKKHVEEQLN - - HGQVYKKAFMQVVEADMIGRGLL	4

[1] The amino acid sequence from residue 162 to 196 in T7 RNAP (or 163 - 197 in T3 RNAP) is shown. Alterations in the sequence are indicated in boldface, dashes indicate deletions in the sequence; multiple amino acids have been deleted and replaced in the RNAPs encoded by plasmids pAR3207 and pBH200.

[2] References are as follows: 1, (Davanloo *et al.*, 1984); 2, (He, 1996); 3, (Lyakhov *et al.*, 1992); 4, this work; 5, (Macdonald *et al.*, 1994); 6, (McGraw *et al.*, 1985).

[3] Encodes a histidine-tagged version of the indicated RNAP. The presence of the amino-terminal histidine tag does not affect the termination properties of the enzyme (He *et al.*, 1997b)

Table II. Termination Efficiencies

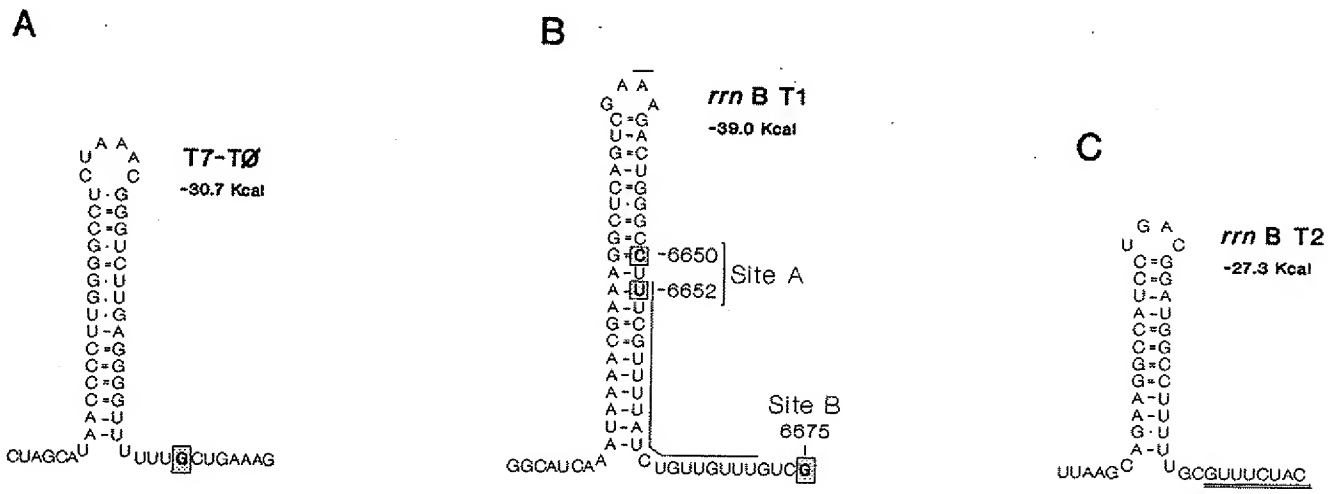
Terminator	RNAP[1]														
	T7												T3		SP6
	WT	K163A	K164A	del163-4	163-9A	K172G	K172L	del172-3	178-193 WIHM	*ins*G181	*ins*G180	R173C	WT	del173-4	WT
TΦ	0.80[2]	0.77	0.78	0.77	0.78	0.79	0.78	0.77	0.72	0.74	0.75	0.75	0.66[3]	*n.d.*	0.64
rrnB T1, Site A	0.65	0.66	0.61	0.66	0.70	0.65	0.65	0.70	0.68	0.69	0.70	0.69	*n.d.*	*n.d.*	*n.d.*
rrnB T2	0.64	0.48	0.51	0.51	0.66	0.63	0.64	0.64	0.60	0.63	0.62	0.62	*n.d.*	*n.d.*	*n.d.*
PTH	0.54	0.41	0.53	0.05	<0.01	0.53	0.54	<0.01	<0.01	<0.01	<0.01	<0.01	0.53	<0.01	0.80
rrnB T1, Site B	0.62	0.66	0.68	0.10	<0.01	0.61	0.63	<0.01	<0.01	<0.01	<0.01	<0.01	*n.d.*	*n.d.*	*n.d.*
CJ	0.29	*n.d*	*n.d*	*n.d*	<0.01	0.28	0.28	<0.01	<0.01	<0.01	<0.01	<0.01	0.30	<0.01	*n.d.*

[1] The identity of the RNAP as shown in Table I.
[2] Calculated as: (termination efficiency) = (termination product) + [(termination product) + (run-off product)] as determined from PhosphorImager™ data. The values represent the average of 2-5 determinations; there is a slight experimental variability (±5%) among experiments.
[3] Data from Macdonald *et al.*, 1993.

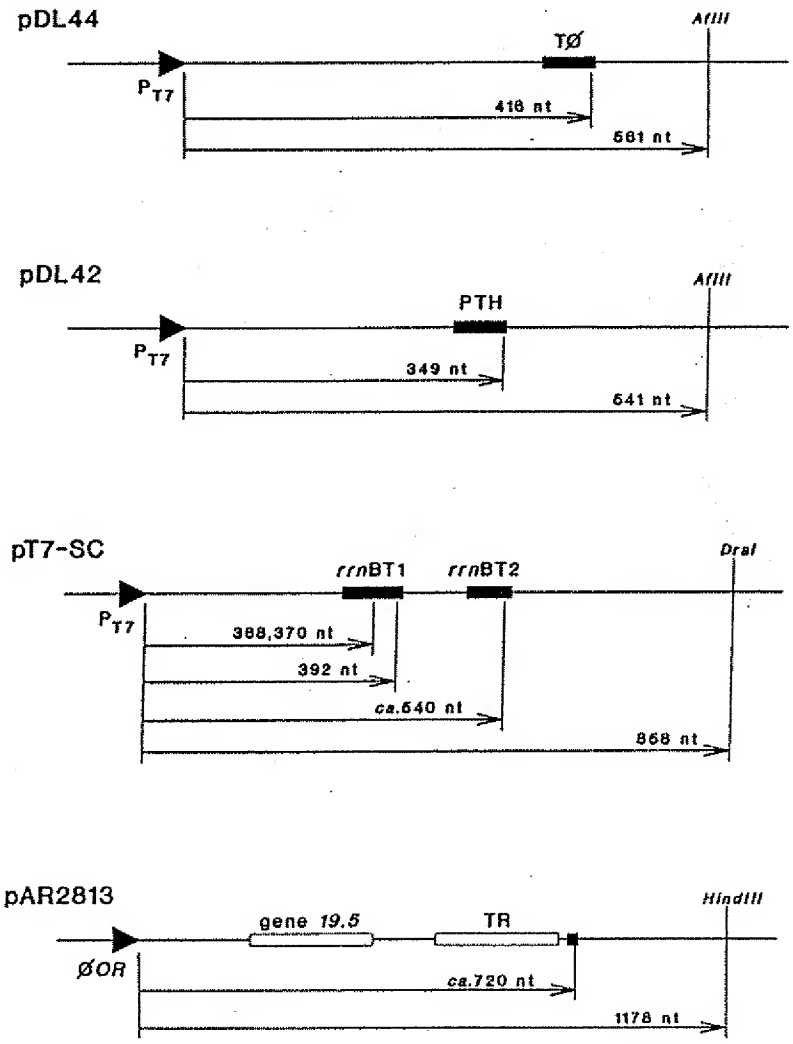
Table III. Effects of T7 lysozyme on termination

Terminator	Lysozyme	RNAP[1]			
		WT	del172-3	X19[2]	BG8[2]
CJ	-	0.26	<0.01	0.72	0.56
	+	0.77	<0.01	0.88	0.71
TΦ	-	0.83	0.81	0.87	0.82
	+	0.84	0.79	*n.d.*	*n.d.*
*rrn*B T_2	-	0.64	0.64	0.62	0.50
	+	0.70	0.67	*n.d.*	*n.d.*
*rrn*B T_1, Site A	-	0.65	0.70	0.95	0.75
	+	0.63	0.73	*n.d.*	*n.d.*
PTH	-	0.54	<0.01	0.79	0.62
	+	0.60	<0.01	*n.d.*	*n.d.*
*rrn*B T_1, Site B	-	0.62	<0.01	0.75	0.70
	+	0.74	<0.01	*n.d.*	*n.d.*

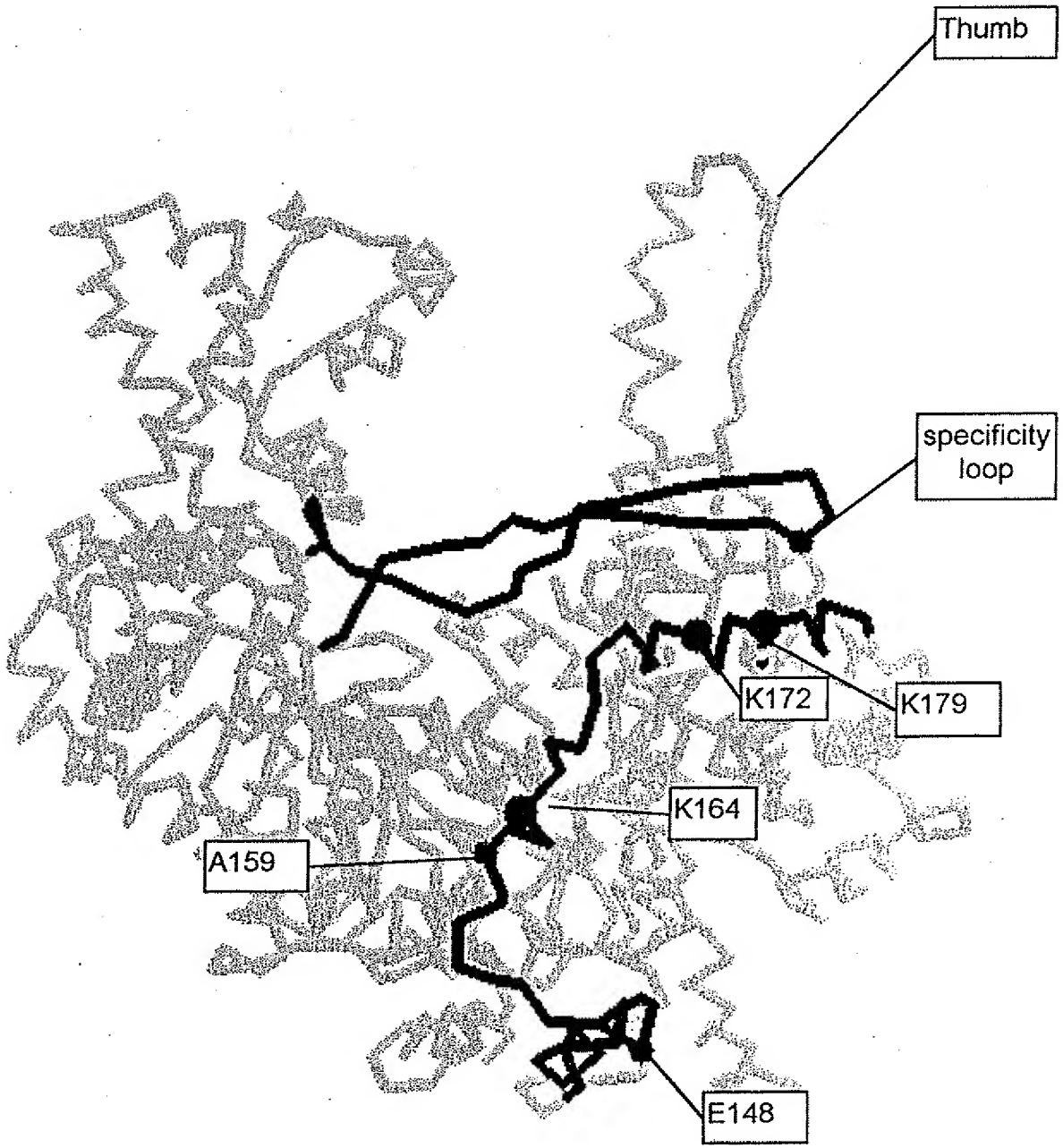
[1] The efficiencies of termination for each RNAP at the termination signal indicated were determined as described in Figure 4 and Table II.
[2] X19 (A465T) and BG8 (A702T) are mutant T7 RNAPs that show increased sensitivity to lysozyme (Zhang & Studier, 1995).


A
T7-TØ
-30.7 Kcal
CUAGCA
UUUGCUGAAAG
B
rrn B T1
-39.0 Kcal
-6650
-6652
Site A
Site B
6675
GGCAUCA
UGUUGUUUGUCG
C
rrn B T2
-27.3 Kcal
UUAAG
GCGUUUCUAC
D
PTH
UCAUGCUUGCCAUCUGUUUUCUUGCAAGUCAGAUGGGA
E
CJ
CUGUGUCCCUAUCUGUUACAGUCUCCUAAAGUAU
TR

DL Fig.1

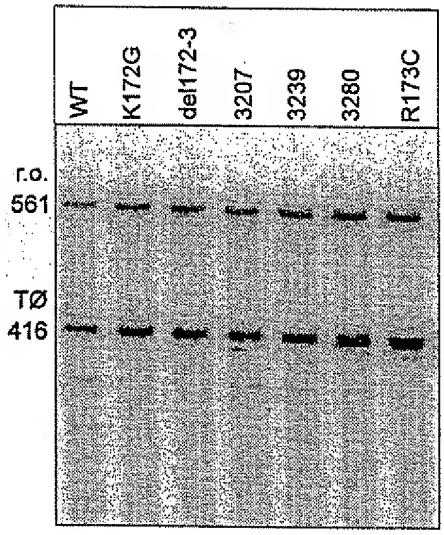

pDL44
TØ
AflII
P_{T7}
416 nt
561 nt
pDL42
PTH
AflII
P_{T7}
349 nt
541 nt
pT7-SC
rrnBT1
rrnBT2
DraI
P_{T7}
388,370 nt
392 nt
ca.640 nt
868 nt
pAR2813
gene 19.5
TR
HindIII
ØOR
ca.720 nt
1178 nt

DL Fig.2

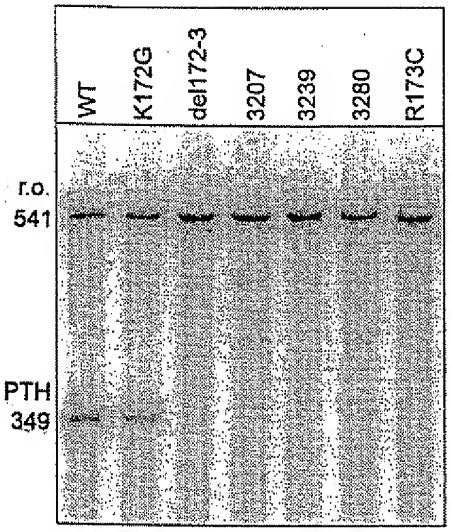


DL Fig 3

A

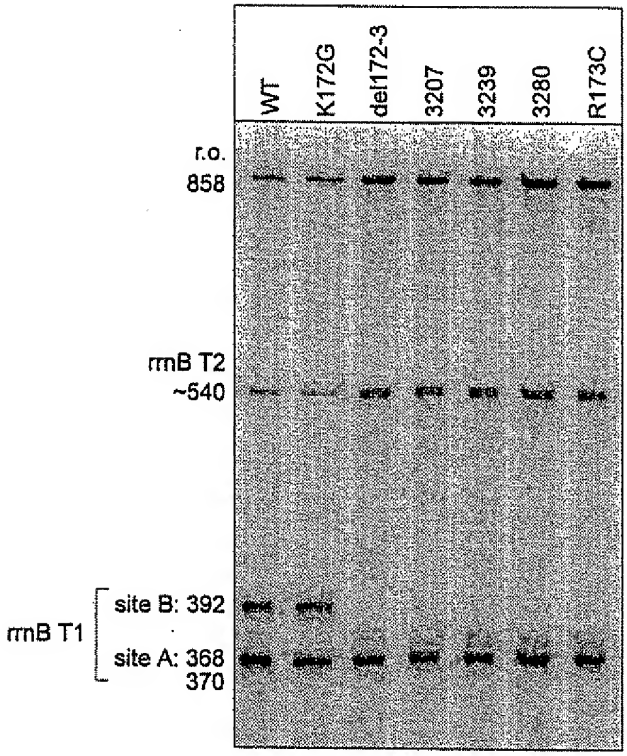

TØ
WT
K172G
del172-3
3207
3239
3280
R173C
r.o.
561
TØ
416

B

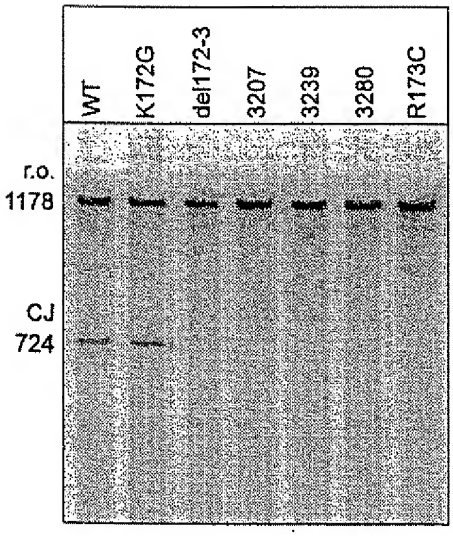

PTH
WT
K172G
del172-3
3207
3239
3280
R173C
r.o.
541
PTH
349

DL Fig.3AB

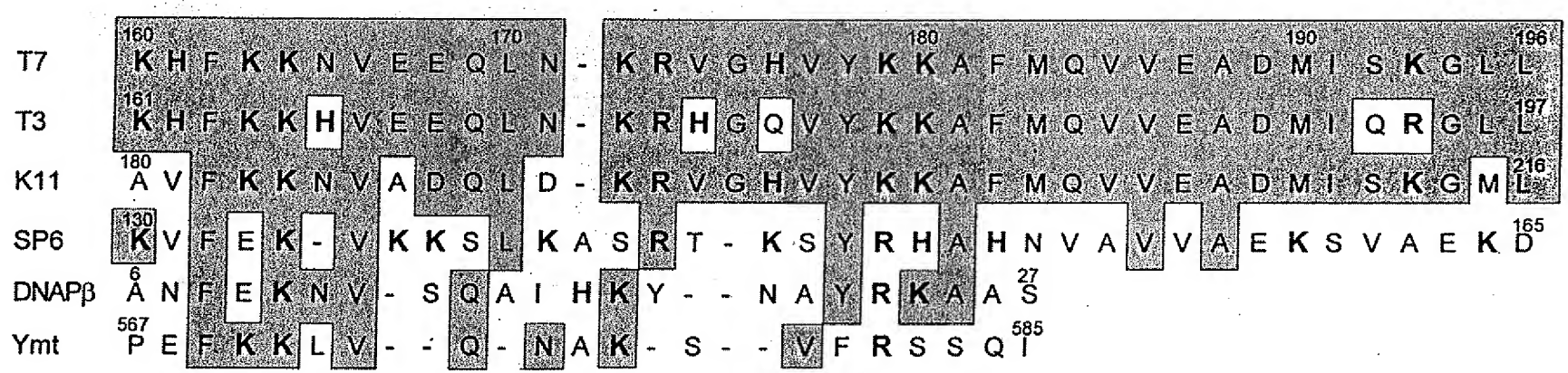
C


rrnB T1,T2
WT
K172G
del172-3
3207
3239
3280
R173C
r.o.
858
rrnB T2
~540
rrnB T1
site B: 392
site A: 368
370

D

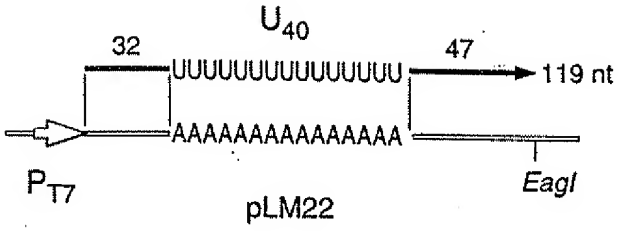

CJ
WT
K172G
del172-3
3207
3239
3280
R173C
r.o.
1178
CJ
724

DL Fig.3CD

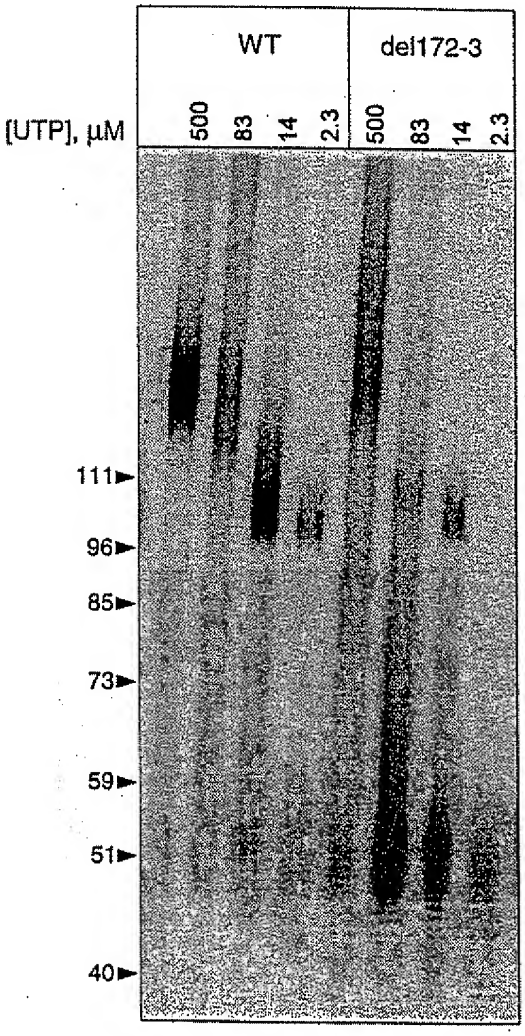

T7 160 K H F K K N V E E Q L N - K R V G H V Y K K A F M Q V V E A D M I S K G L L 196
T3 161 K H F K K H V E E Q L N - K R H G Q V Y K K A F M Q V V E A D M I Q R G L L 197
K11 180 A V F K K N V A D Q L D - K R V G H V Y K K A F M Q V V E A D M I S K G M L 216
SP6 130 K V F E K - V K K S L K A S R T - K S Y R H A H N V A V V A E K S V A E K D 165
DNAPβ 6 A N F E K N V - S Q A I H K Y - - N A Y R K A A S 27
Ymt 567 P E F K K L V - - Q - N A K - S - - V F R S S Q I 585

DL Fig.5

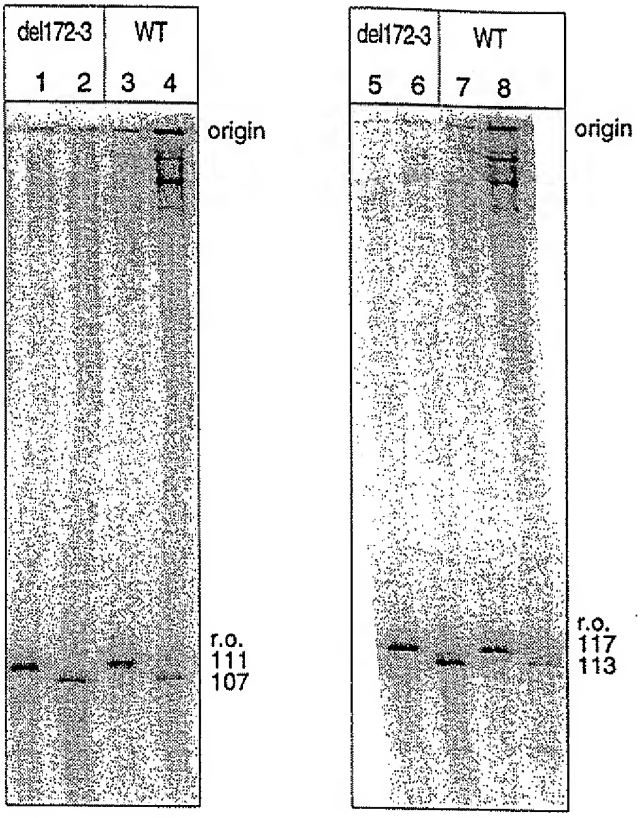
A


U40
32
47
UUUUUUUUUUUUUUU
119 nt
AAAAAAAAAAAAAAA
PT7
pLM22
EagI

B


WT
del172-3
[UTP], µM
500
83
14
2.3
500
83
14
2.3
111
96
85
73
59
51
40

DL Fig.6



Lane	Enzyme	End
1,3	Bsp120I	G|GGCCC -3' CCCGG|G -5'
2,4	ApaI	GGGCC|C -3' C|CCGGG -5'
5,7	Acc65I	G|GTACC -3' CCATG|G -5'
6,8	KpnI	GGTAC|C -3' C|CATGG -5'

DL Fig.7